

## Detection and Molecular Characterization of *gyrA* and *gyrB* Genes of MDR-*Salmonella typhi* Isolated from Clinical Sample in Sudan

Kyakonye Yasin<sup>1</sup>, Mwatasa H Mwanganyuma<sup>1</sup>, Ftum M Amir<sup>1</sup>, Muzamil M Abdel Hamid<sup>2</sup>, Hisham N Altayb<sup>3</sup>, Mohamed M Hassan<sup>4</sup> and Mohamed A Hussain<sup>1\*</sup>

<sup>1</sup>Department of Pharmaceutical Microbiology, International University of Africa, Sudan

<sup>2</sup>Department of Parasitology and Medical Entomology, Institute of Endemic Diseases, Khartoum University, Sudan

<sup>3</sup>Department of Microbiology, Faculty of Medical Laboratories Sciences, Sudan University of Sciences and Technology, Sudan

<sup>4</sup>Faculty of Medical Laboratory Sciences, University of Medical Science and Technology, Sudan

\*Corresponding Author: Mohamed A Hussain, Department of Pharmaceutical Microbiology, International University of Africa, Sudan.

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### Abstract

Multidrug resistance has contributed widely to increase in morbidity and mortality as it leads to treatment failures and compromising empiric therapy in the management of severe acute infections. In this study, we characterize the molecular basis of resistance to Ciprofloxacin of a MDR-*Salmonella typhi*.

Antimicrobial susceptibility testing was done on pre-serotyped clinical samples to screen for MDR-*S. typhi*. DNA was extracted using phenol-chloroform method followed by PCR to amplify *gyrA* and *gyrB* genes. DNA amplicons were sequenced and analyzed using bioinformatics techniques.

Alignment of nucleotide sequence with reference sequence revealed non-synonymous deleterious mutations at positions 248 and 259 from C->T and G-A with multiple synonymous mutations at positions 255, 273, 300, 333, 468, 570 and 594 from C->T, C->T, T->C, T->C, G->C, C->T and T->C respectively. Alignment of translated amino acid sequence with reference sequence showed amino acid substitution of serine to leucine and aspartate to asparagine at positions 83 and 87 respectively. A novel deleterious mutation was found in *gyrB* gene sequence of G->A at position 520 and resulted in amino acid substitution from glutamate to lysine at position 174.

We report MDR-*Salmonella typhi* whose *gyrB* gene sequence contained a novel deleterious mutation and *gyrA* gene possessed two deleterious point mutations confirming to previous reports on complete Ciprofloxacin resistance.

**Keywords:** MDR-*S. typhi*; Novel Mutation; Sudan

### Introduction

Global rates of microbial resistance and multidrug resistance is on a rise [1]. The World Health Organization (WHO) defines antimicrobial resistance as resistance of a microorganism to antimicrobial drug that was originally effective for the treatment of infection caused by it [2]. Resistance to typhoid treatment puts more disease burden especially to low income countries where use of newer expensive drugs may be limited, thus contributing to a rise in morbidity and mortality rates [3]. Typhoid fever is a systemic infection transmitted by consumption of contaminated food or water [4]. Multidrug resistant typhoid fever (MDRTF) is resistant to treatment with first line drugs like Chloramphenicol, Ampicillin and Co-trimoxazole [5]. The trend of antimicrobial resistance estimated in reports from Indonesia, Taiwan and Vietnam indicated that majority of the isolates from Bangladesh and Vietnam were MDR and belonged to a wide spread haplotype H58 clone. Majority of the isolates from Indonesia and Taiwan were all susceptible to the tested antimicrobial agents. All MDR isolates from Vietnam incorporated IncH11 plasmid and only 15% of the total isolates from Bangladesh carried such plasmid, 82% and 40% of the isolates from Bangladesh were resistant to various concentrations of Nalidixic acid and Ciprofloxacin respectively [6]. Reports from India

between January 2009 and June 2013 indicated a decline in MDR by 18.2% in *S. typhi* and absence of resistance in *S. paratyphi* A. however; there was an observed increase in the resistance to fluoroquinolone antimicrobial agents and it was mediated by a non-conjugative and non IncH11 plasmid in over 71.4% of all the MDR strains [3]. Resistance to quinolones was associated with mutations in quinolone resistant determining region (QRDR) of *gyrA* gene at position 83 from serine to phenylalanine, the isolates that were found resistant to both Nalidixic acid and Ciprofloxacin contained a double mutation of serine 83 to phenylalanine and aspartate 87 to asparagine. Additionally, mutations of serine 80 to isoleucine in *parC* gene also contributed to resistance to quinolone antibiotics [7]. WHO's global report indicated that antimicrobial resistance is no longer a prediction for the future and thus needs coordinated action to limit its spread [2]. WHO puts emphasis on infection prevention to limit antimicrobial use, better hygiene, access to clean water, infection control in health facilities and vaccination as means of minimizing antimicrobial resistance [2]. It is recommended to use Ciprofloxacin and Ceftriaxone in the treatment of MDRTF [5] however; treatment of typhoid fever should be done with a single antimicrobial agent for a period of 10 - 14 days following antimicrobial susceptibility tests [8]. With the increasing rise of treatment failures observed in major health centers in Sudan, we aimed at detecting and characterizing the molecular basis of resistance to Ciprofloxacin a widely prescribed antibiotic for typhoid treatment.

## Materials and Methods

### Antimicrobial susceptibility tests

Pre- screened and serotyped clinical isolates of typhoid-causing *Salmonella* Species were collected from diagnostic laboratories in Khartoum state and cultured on MacConkey agar and Xylose lysine deoxycholate (XLD) prepared according to manufacturer's instructions (HiMedia LTD India). Growth pattern was inspected and pure colonies were obtained for biochemical tests and antimicrobial susceptibility tests. Antimicrobial susceptibility testing was done according to clinical and laboratory standards institute (CLSI) [9]. The antimicrobial agents tested were, Ciprofloxacin 5 mg, Cotrimoxazole 25 mg, Ceftriaxone 30 mg, Ceftazidime 30 mg, Meropenem 10 mg and Chloramphenicol 30 mg.

### DNA Extraction and PCR

DNA extraction was done for the MDR isolate using Phenol-chloroform method. DNA quantification was done by Nano drop spectrophotometer (ND1000) and stored at -20°C until PCR was done. The primer set of *gyrA* F 5' agtacgtgggcaatgtgtt3', R 5'gcccttcaatgctgatgtct3' product size 691bp and *gyrB* F 5'caagattaaatgagcgagaacg3', R 5'tgcggaatgtgttgtaaa3' product size 700bp were designed using primer 3plus online software [10] and synthesized by Macrogen company, Soul Korea. PCR was carried out using thermal cycler (Senso-Quest, Germany) using the primer sets and a prepared master mix (Maxime RT premix kit 5 µl iNtRON BIOTECHNOLOGY, Seongnam, Korea) with a total volume of 25 µl (22 µl of de ionized sterile water, 1 µl of each primer and 1 µl of DNA template). PCR mixture was subjected to initial denaturation step at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 45 seconds, primer annealing at 55°C for 45 seconds, followed by step of elongation at 72°C for 60 seconds.

### Visualization of PCR products

Electrophoresis was done using 1.5% agarose gel (Caisson, USA at 100V and 30mA, and the product was visualized under U.V transilluminator (BioDoc-It UVP, Cambridge UK), the size of PCR product was estimated by a standard 100bp DNA ladder (INTRON).

### Sequencing of PCR products

High quality DNA purification and sequencing was done by Macrogen Company (Soul, Korea).

### Bioinformatics analysis

Sequence cleaning and nucleotide blast were done using Finch TV software version 1.4.0 [11].

Visualization of DNA sequence and multiple sequence alignment with retrieved highly similar sequences from NCBI to detect mutation was done using Bio Edit software [12]. Translation of nucleotide sequence was done using NCBI EMBOSS transeq online translation tool [13]. SIFT online software was used to predict the impact of mutations on amino acid primary sequence [14]. The impact of mutations on

the protein stability was predicted using I-mutant software [15]. The 3D structure of the protein was predicted using CPH models [16], and visualized using chimera software version 1.8 [17]. Comparison of amino acid properties was done using ExPASy pI/Mw computing tool [18].

## Results

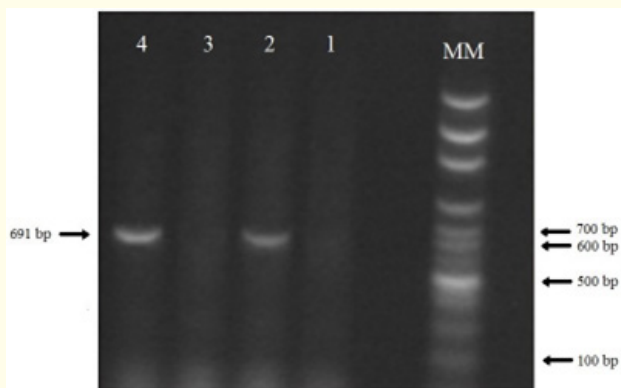
The bacteria isolate was resistant to Ciprofloxacin, Co-trimoxazole, Ceftriaxone, and Ceftazidime. However, it was susceptible to Chloramphenicol and Meropenem. Table 1 shows qualitative and quantitative antimicrobial susceptibility tests. Figure 1a and 1b shows the DNA bands Visual picture for the two amplified subunits of topoisomerase gene (*gyrA* and *gyrB*). The nucleotide sequence of *gyrA* gene was aligned with reference sequence of *E. coli* str. K-12 substr. MG1655 accession number. NC\_000913.3 NCBI, and mutations were detected at positions 248, 255, 259, 273, 300, 333, 468, 570 and 594 from C->T, C->T, G->A, C->T, T->C, T->C, G->C, C->T and T->C respectively. Alignment of the amino acid sequence with reference sequence obtained from UniProtKB/Swiss-Prot: P0AES4 showed two amino acid substitutions of serine to leucine and aspartic acid to asparagine at positions 83 and 87 respectively which corresponded to mutations at positions 248 (C->T) and 259 (G->A) in the nucleotide sequence (Figure 2). The rest of the nucleotide substitutions resulted in no changes in amino acid sequence thus being synonymous mutations. SIFT analysis showed a highly damaging effect with amino acid asparagine at position 87 but the substitution of leucine at position 83 was benign. I-mutant analysis predicted a significant decrease in stability of protein 3D structure due to asparagine substitution with DDG value prediction -0.44 Kcal/mol. however, the serine to leucine substitution produce a negligible decrease in energy with DDG value prediction = 0.06 Kcal/mol. There is an observed difference in structural conformation due to substitution of leucine amino acid sequence in the predicted 3DS using chimera software but no such conformation changes were associated with asparagine substitution at position 87 in the amino acid sequence (Figure 3). The theoretical isoelectric point and molecular weight of translated amino acid was pI/Mw: 5.28/20158.02 for the mutant sequences compared to wild type sequence pI/Mw: 5.13/20132.92.

A novel mutation was detected in *gyrB* nucleotide sequence due to base pair substitution from G->A at position 520 (Figure 4), nucleotide sequence was aligned based on *E. coli* str. K-12 substr. MG1655, accession no. NC\_000913.3 NCBI and amino acid sequence from UniProtKB/Swiss-Prot: P0AES6, this mutation produced a change in amino acid from glutamate to lysine at position 174 which was deleterious as predicted by SIFT analysis and confirmed by I-mutant analysis with a decrease in energy of -0.56 Kcal/mol. in the protein 3DS. No significant structural conformation changes were associated with this substitution on the protein 3D structure (Figure 5). The resultant protein isoelectric point and molecular weight for the mutant and wild type were compared and that of mutant had a raise in isoelectric point pI = 5.51 compared to that of wild type pI = 5.24 with a slight reduction in molecular weight Mw: 17677.96 compared to that of wild type Mw = 17678.90.

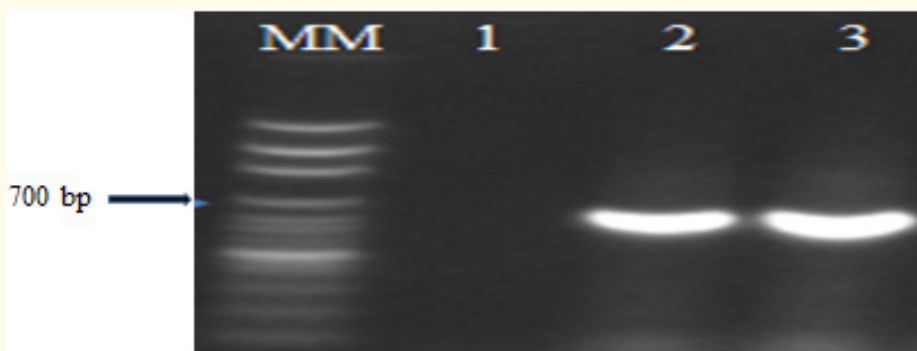
Isolate	Antimicrobial zone of inhibition (mm)					
	CIP 5	STX 25	CRO 30	CAZ 30	C 30	MEM 10
<i>S. typhi</i>	0,R	0,R	8,R	14,R	28,S	40,S

**Table 1:** The qualitative and quantitative antimicrobial susceptibility test result of MDR-*S. typhi* isolate from Sudan.

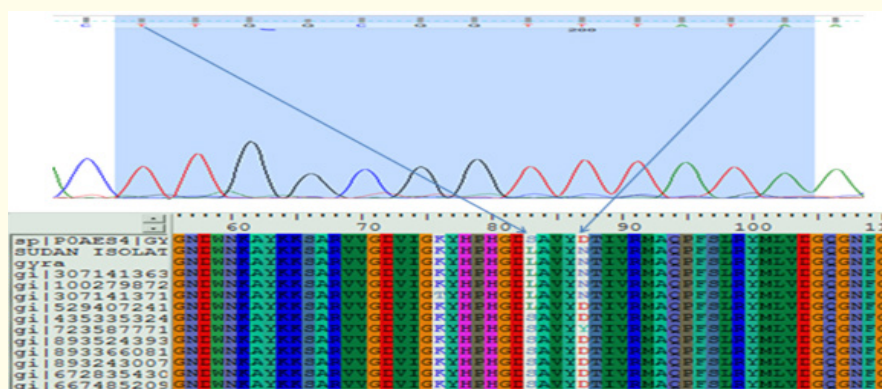
Abbreviations; S: sensitive, I: intermediate sensitive, R: resistant, CIP5: Ciprofloxacin 5 mg, STX25: Cotrimoxazole 25 mg, CRO30: Ceftriaxone 30 mg, CAZ30: Ceftazidime 30 mg, C30: Chloramphenicol 30 mg and MEM10: Meropenem 10 mg.



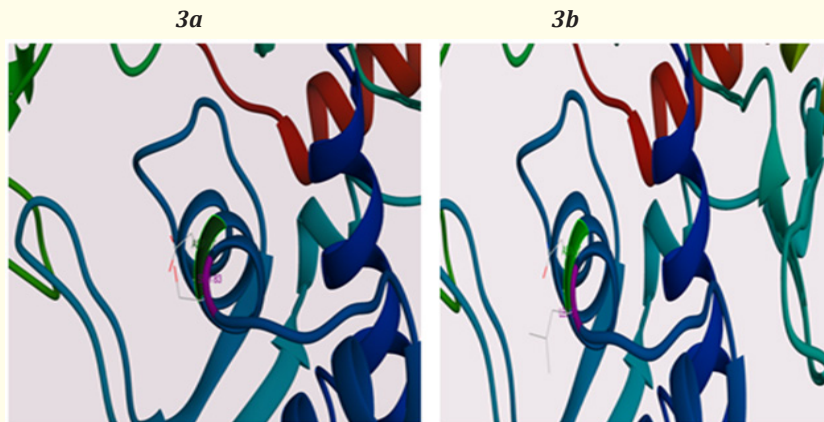
**Figure 1a:** The visual picture PCR products for *gyrA* gene run on 1.5% agarose gel, MM is 100Mw DNA ladder, 1 and 3= negative control, 2= positive control and 4 corresponds to 691bp of amplified *gyrA* gene band.



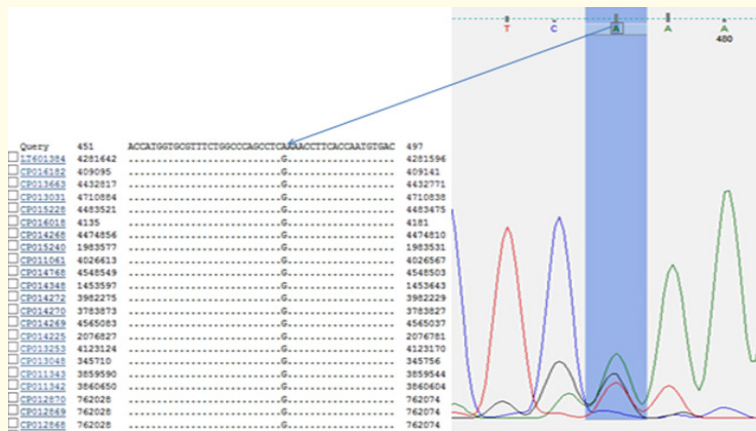
**Figure 1b:** The visual picture of PCR product for *gyrB* gene run on 1.5% agarose gel, MM= 100Mw DNA ladder, 1= negative control, 2= positive control and 3 corresponds to 700bp of amplified *gyrB* gene band.



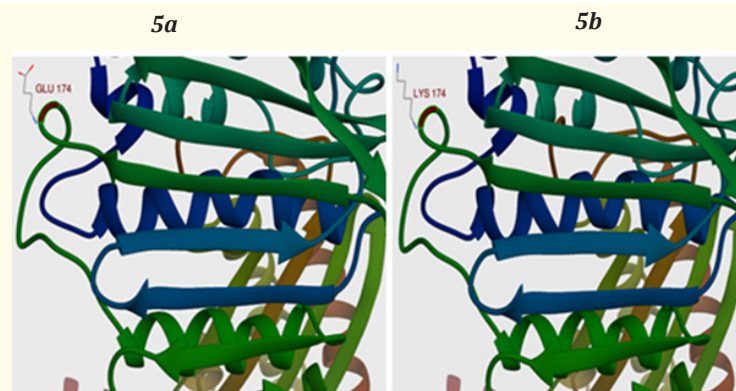
**Figure 2:** A chromatogram showing two non-synonymous deleterious mutations in *gyrA* gene sequence at positions 248 C->T and 259 G->A viewed in Finch TV coupled to multiple amino acids sequence alignment showing the resultant changes in amino acids serine 83 to leucine and aspartate 87 to asparagine visualized in bio edit software.



**Figure 3:** A chimeric visual picture showing comparison of the wild type (3a) and mutant (3b) amino acid 3D structural conformation with the substitution of serine 83 to leucine and aspartate 87 to asparagine.



**Figure 4:** A chromatogram of *gyrB* gene sequence showing a novel nucleotide mutation at position 520 G->A viewed in Finch TV with a corresponding online visual confirmation picture of pairwise alignment.



**Figure 5:** A comparison of structural conformation changes in the wild type 5a and mutant 5b due to amino acid substitution glutamate 174 to lysine in amino acid sequence of *gyrB* gene viewed in chimera.



## Discussion

In current study, we found mutations in *gyrA* gene that led to subsequent changes in amino acids at position 83 from serine to leucine, this change was associated with structural conformation changes in the protein 3DS as predicted by CPH models and Chimera software but no significant changes in isoelectric properties and energy of the resultant amino acid sequence were observed due to this substitution, this amino acid substitution was not deleterious as per SIFT prediction and I-mutant, however it has been reported as being responsible for Nalidixic acid resistance and reduction in Ciprofloxacin sensitivity in *S. typhi*. A further amino acid change at position 87 from aspartate to asparagine was observed and was predicted as being deleterious by SIFT analysis and produced a significant decrease in energy of the protein 3DS as predicted by I-mutant online software, it was also associated with an increase in protein isoelectric properties and molecular weight, however it was not associated with significant structural conformation changes. These observed changes in protein structural conformation as well as changes in physical properties of the resultant protein explain the mechanism of complete resistance to Ciprofloxacin as reported in previous findings by Gaid [19] and Sreedharan [20]. A novel nucleotide substitution in *gyrB* gene produced a change in amino acid from glutamate to lysine which was deleterious as predicted by SIFT analysis and I-mutant and produced a decrease in energy with DDG value prediction = -0.56 Kcal/mol. in the protein 3DS. This nucleotide substitution in *gyrB* could be contributing significantly to complete Ciprofloxacin resistance observed in current study.

Resistance to ciprofloxacin was mediated through chromosomal mutations in the quinolone resistance determining region, screening for MDR isolates showed an observed shift of MDR from first line drugs to newer antimicrobial agents like third generation Cephalosporin and fluoroquinolones antibiotics, this observation agrees with reports from Bangladeshi and India. The prevalence of MDR-*S. typhi* continues to rise across the globe especially to the newly developed and widely used antimicrobial agents, which further increases incidence of clinical failures with empirical therapy leading to rise in morbidity, mortality as well as increments in healthy expenditure especially in the developing parts of the world. The detection of a complete Ciprofloxacin resistant strain of *S. typhi* from clinical samples stretches the fact that MDR-*S. typhi* has spread across many parts of the world and calls for establishment of regional surveillance programs in the effort to limit its spread and the resultant clinical consequences.

## Conclusion

A MDR strain of *Salmonella typhi* isolated from clinical settings in Sudan has been reported; the isolate possessed reported mutations in its *gyrA* gene and a novel mutation in *gyrB* gene that could be significantly contributing to fluoroquinolones resistance.

## Declarations

All authors declare to no competing interest towards this research study.

## Sequence Data

Sequence data was deposited to National Center for Biotechnology Information (NCBI) with accession number KX702131 for *gyrA* gene sequence and KX702131 for *gyrB* gene of a MDR-*S. typhi* isolate from Sudan.

## Ethics Statement

Ethical clearance was obtained from Ethical committee of the institute of endemic diseases, University of Khartoum.

## Acknowledgement

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